IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

		Docket No. 04-100
Applicant: King et al	)	
	)	
Serial No.: 10/810,829	)	Art Unit 1651
	)	
Filed: 03/29/2004	)	Examiner:
	)	Fernandez, Susan
For: ELECTRODES COATED WITH TREATING AGENT AND USES THEREOF	)	

DECLARATION OF ALAN KING UNDER RULE 132

Mail Stop AF
Commissioner for Patents
P. O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, Alan King, hereby declare:

1. I am a Veterinarian and an Immunologist specializing in the areas of Vaccines and Electroporation.

2. I am a licensed Veterinarian in the State of Maryland.

3. I have received Education as indicated on page 1 of a copy of my CURRICULUM VITAE incorporated in this Declaration as Appendix A.

4. I have had Professional Positions listed on page 1 and 2 of the CURRICULUM VITAE.

5. I am either a sole author or a co-author of the published articles listed on pages 2 to 4 of the CURRICULUM VITAE.

6. I am a joint inventor of U. S. Patents listed on page 4 of the CURRICULUM VITAE.

7. Since 1998 to the present, I am Chief Scientific Officer of Cyto Pulse Sciences, Inc. in Glen Burnie, MD 21061.

8. I know joint inventor Richard Walters who is the President of Cyto Pulse Sciences, Inc. which manufactures the Pulse Agile(TM) system which provides nonperiodic electrical pulses for carrying out electroporation and electrofusion.

9. I have reviewed U. S. Patent No. 5,103,837 of Weidlich et al, and the U. S. Patent No. 6,009,347 of Hoffman and the following is my professional analysis of Weidlich et al (5,103,837) and Hoffman (6,009,347)

[Note: The references cited in the analysis below are contained in Appendix B]

Weidlich (US 5,103,837) :

The only independent claim in patent 837 states (Appendix B, page 1):

1. An electrode comprising:
   a) an implantable, porous, stimulating electrode having a surface bearing a thin coat of a hydrophilic polymer; and
   b) an anti-inflammatory steroid encapsuled by said polymer, whereby diffusion of said anti-inflammatory steroid after implantation into surrounding tissue prevents growth of connective tissue and thus prevents a rise in stimulation threshold of said electrode.

The Weidlich electrode is specifically designed to improve the function of cardiostimulatory electrodes by providing an anti-inflammatory deposition of steroids. The delivery of steroids is by passive diffusion and not by electrical means. The specification states (Appendix b, page 2) "After the electrode has been exposed to body fluids (I.E. after implantation), the steroid is gradually released or eluted"

The body is composed of two fluid containing compartments, intracellular and extracellular. This is more clearly stated in Review of Medical Physiology by Ganong (2005) Chapter 1 (Appendix B, page 4) "The General and Cellular Basis of Medical Physiology" as follows:

"The cells that make up the bodies of all but the simplest multicellular animals both aquatic and terrestrial, exist in an internal sea of extracellular fluid (ECF) enclosed within the integument of the animal. From this fluid, the cells take up O_2 and nutrients; into it they discharge metabolic waste products. The ECF is more dilute than present day sea water but its composition resembles that of the primordial oceans in which, presumably, all life originated." (Continued next paragraph)

"In animals with a closed vascular system, the ECF is divides into two components: the interstitial fluid and the circulating blood plasma. The plasma and the cellular components of the blood, principally red blood cells, fill the vascular system, and together they constitute the total blood volume. The interstitial fluid is that part of the ECF that is outside the vascular system, bathing the cells."

The interstitial fluid is the fluid that bathes both the Cyto Pulse electrode and the 837 electrode. This is where the similarity ends. The 837 electrode is coated with a steroid that elutes from the electrode over time. By definition, the first compartment that receives the steroid is the interstitial fluid. From that point, the steroid must get into cells to be effective. Steroids are unique in that their receptors are inside of a cell.

According to Goodman and Gillman's "The Pharmacological Basis of Therapeutics" 11th Edition, Chapter 59 (Appendix B, page 6), "The glucocorticoid receptor resides predominantly in the cytoplasm in an inactive form until it binds glucocorticoids". To get to its receptor, the glucocorticoid (steroid) diffuses through the cell membrane. It does not have to be actively delivered to the cytoplasm.

DNA or other polynucleotides, on the other hand must be actively delivered into cells in order to move from the interstitial compartment to the intracellular compartment. The 10/810,829 application teaches electroporation as a means to move the DNA from the interstitial compartment to the intracellular compartment. This is a step not taught in the 837 patent.

The ability of a substance to traverse the cell membrane from an interstitial compartment to an intracellular compartment is a function of the size and charge of the molecule. This is well illustrated in Chapter 15, figure 15-1 of "Molecular Cell Biology" by Darnell (Appendix B, page 9). According to this table, water, gasses, and small uncharged polar molecules readily cross the cell membrane while large uncharged polar molecules, ions and charged polar molecules do not readily cross the cell membrane. Steroids are part of the group that readily cross the cell membrane (small uncharged molecules) while DNA and other polynucleotides do not readily cross the cell membrane since DNA is both large and charged.

Thus the examiner's claim that "the steroid is delivered into biological cells in the tissues penetrated by the electrode by the electric field applied to the penetrated tissues" is not true. It merely diffuses into the cells.

The specifications and the claim in the 837 patent describe an electrode with a steroid on the surface for the purpose of preventing "growth of connective tissue". The electrode is used in a pacemaker. The application of the electrical pulses and the elution of the steroid in the 837 patent are temporally disconnected. That is, the steroid is continuously eluted from the surface while the electrical pulses are only delivered if the heart malfunctions. Alternatively, the elution of DNA from a coated needle and the application of electrical pulses taught in the 10/810,829 application are temporally related. The electrical pulses are applied immediately after insertion and at no other time.

The Weidlich 837 patent requires a polymer covering on the electrode to hold the steroid (claim 1). The purpose of the polymer is to provide a timed release of the steroid. Their summary of the invention clearly describes a "dose release of the steroid". The exact paragraph 2 of the summary of the invention follows:

"The porous electrode surface of the electrode of the present invention is provided with a thin layer of a polymeric plastic, into which is inserted a steroid that diffuses from this layer into the adjoining tissue. In this manner, the inflammatory process is suppressed and the process by which the electrode becomes incorporated in the tissues of the heart muscle is abetted. By means of the spatially uniform, dosed release of the steroid in the region around the electrode head, the surrounding tissue is supplied with steroid uniformly over a short distance. Consequently, the growth of connective tissue is reduced and thus the postoperative rise in the stimulation threshold is lessened. The steroid is then available when needed immediately after implantation. Within as few as two days following implantation, the steroid is nearly completely eluted. As a result of the measures according to the invention, the porous surface of the electrode is protected from impurities. However, the porous electrode surface suffers no loss of capacitance."

Hoffman (6,009,347):

The summary of the invention for the Hoffman patent follows:

"In accordance with a primary aspect of the present invention an electrode template apparatus, comprises a three dimensional support member having opposite surfaces, a plurality of bores extending through said support member, a plurality of conductors on said member separately connected to contacts in said plurality of bores, a plurality of electrodes selectively insertable in said plurality of bores so that each conductor is connected to at least one electrode, and means for connecting said electrode template to a power supply."

These features are incorporated in various ways in three independent claims.

In this invention a plurality of needles are selectively inserted into a plurality of bores. This provides a multitude of configurations. In other words there is no fixed number or length of needles.

The Hoffman electrode was designed for electroporation as is stated in the first sentence of the background section: "The present invention relates to electroporation and pertains particularly to an apparatus with connective electrode template for electroporation therapy." This is supported throughout the specifications.

For electroporation to work, the material must be in the applied electric field prior to application of the electrical pulses (see Table 1 in Wolf et al, Control by pulse parameters of electric field-mediated gene transfer in mammalian cells, Biophysical Journal, 66:524-531 (Appendix B, page 11)). The only means mentioned to deliver material for electroporation is by injection. This is illustrated in dependent claim #9 as follows:

"9. An electrode apparatus according to claim 8 wherein at least one of said plurality of electrodes have a tubular configuration for injection of molecules into tissue."

Combining inventions:

It is not possible to combine Weidlich and Hoffman to make the Cyto Pulse application invention.

An essential part of the Weidlich coating is a polymer coating impregnated with steroid for a timed release of the steroid. If this coating were to be placed on the Hoffman device, the steroid would not be delivered in time to be electroporated into cells. The steroid because of its small molecule and non-ionic nature crosses cell membranes without the need of electroporation. If DNA were substituted for the steroid the diffusion time would be exponentially longer (due to the large size of the DNA) and DNA is not mentioned in the Weidlich patent.

If the Weidlich coating were used on the Hoffman device and DNA was substituted for the steroid, the electroporation pulses would have to be given days later. In addition, because of DNAse in the skin, there would never be enough DNA around the electrode for electroporation into the cells.

The dose is another factor that would prevent the combination of Weidlich and Hoffman. The dose on the Weidlich electrode is fixed and slowly released. The dose on the Hoffman device is fixed and rapidly released. However, the doses are released

differently and the combination of the two devices would result in a variable dose. This is because, if the dose on the Hoffman device were coated onto the needles like the Weidlich device, the dose would depend upon how many needles were used with the Hoffman electrode and how deep the electrodes were inserted. Since both of these are variable on the Hoffman electrode, the dose would then become variable. A pre-determined, fixed dose is desirable in the Cyto Pulse invention.

A summary of the similarities and differences of the inventions is shown in the following table:

Feature	Weidlich	Hoffman	Combined*	Cyto Pulse
Molecule size	Small	Small/Large	Small	Large
Rate of release	Slow	Fast	Slow	Fast
Delivery-Pulse Time#	Days to weeks	Seconds	Days to weeks	Seconds
Porous coating	Yes	No	Yes	No
Dosing Means	Coating	Injection	Coating	Coating
Electrode number	2	variable	variable	fixed
Electroporation	No	Yes	Yes	Yes
Dry coating	Yes	No	Yes	Yes

*Combined means the coating features of the Weidlich device and the needle structure and electroporation of the Hoffman device.
Delivery-Pulse Time means the time from the start of drug delivery to delivery of last pulse

Clearly a combined Weidlich-Hoffman device does not make a Cyto Pulse invention.

APPENDIX A

CURRICULUM VITAE OF ALAN KING

Curriculum Vitae

Alan D. King, D.V.M., Ph.D.
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Place of Birth: Birmingham, Alabama
Date of Birth: 7 June 1947

University Education:

1990	Ph.D., Cornell University, Ithaca, New York Subject: Immunology. Dissertation topic: The Role of CD8 cells in Immunity to *Listeria monocytogenes*.
1978	D.V.M., Auburn University, School of Veterinary Medicine, Auburn, Alabama
1973	B.S., University of Alabama, Tuscaloosa, Alabama. Major: Psychology, Minor: Chemistry

Military Education:

1992	Command and General Staff College (3 year correspondence course)
1984	Non-Appropriated Fund Management, Garmish, West Germany, Duration: 40 hours.
1983	Officers Advanced Course (2 year correspondence course)
1980	Veterinary Food Inspection, Fort Sam Houston Texas, Duration: 6 weeks.
1980	Officers Basic course, Fort Sam Houston, Texas, Duration: 4 weeks.

Professional Positions:

1998- Present	Chief Scientific Officer, Cyto Pulse Sciences, Inc.
1992-1998	Research Scientist, Department of Virus Diseases, Walter Reed Army Institute of Research.
1990-1992	Assistant Chief, Department of Virology, Armed Forces Research Institute

of Medical Sciences, Bangkok, Thailand

1986-1990 Graduate Research Assistant, Cornell University, James A. Baker Institute for Animal Health, Ithaca, NY

1984-1986 Veterinary Service Officer, U. S. Army Medical Research Institute of Infectious Diseases, Fort Detrick, MD

1982-1984 Executive Officer, 483rd Medical Detachment, Munich Germany.

1980-1981 Chief, Veterinary Activity, Fort Monmouth, NJ

1978-1980 Veterinarian, Parkway Animal Hospital, 8560 Arlington Expressway, Jacksonville, FL.

Publications:

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Patents:

United States Patent No. 6,916,656 Non-linear amplitude dielectrophoresis waveform for cell fusion Richard E. Walters, Derin C. Walters, Alan D. King U. S. 12 Jul 2005

United States Patent 6,713,291 Electrodes coated with treating agent and uses thereof Allowed March 30, 2004 King; Alan D. Walters; Richard E. Filed August 3, 2001

United States Patent 6,653,114 Method and apparatus for treating materials with electrical fields having varying orientations Walters; Richard E.; King; Alan D.; Walters; Derin C. November 25, 2003

United States Patent 6,603,998 Delivery of macromolecules into cells King; Alan D.; Walters; Richard E. August 5, 2003

United States Patent 6,514,501 Recombinant vaccine against dengue virus Kelly; Eileen P., King; Alan D. February 4, 2003

United States Patent 6,117,660 Method and apparatus for treating materials with electrical fields having varying orientations Walters; Richard E. King; Alan D. ;Walters; Derin C. September 12, 2000

United States Patent 6,074,865 Recombinant dengue virus DNA fragment Kelly; Eileen P., King; Alan D. June 13, 2000

United States Patent 6,010,613 Method of treating materials with pulsed electrical fields Walters; Richard E. King; Alan D., January 4, 2000

APPENDIX B

REFERENCES CITED IN ANALYSIS
OF
Weidlich et al (5,103,837) and Hoffman (6,009,347)

4692336	September 1987	Eckenhoff et al.
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Foreign Patent Documents

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Other References

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Primary Examiner: Cohen; Lee S.
Attorney, Agent or Firm: Kenyon & Kenyon

Claims

What is claimed is:

1. An electrode, comprising:

a) an implantable, porous, stimulating electrode having a surface bearing a thin coating of a hydrophilic polymer; and

b) an anti-inflammatory steroid encapsulated by said polymer, whereby diffusion of said anti-inflammatory steroid after implantation into surrounding tissue prevents growth of connective tissue and thus prevents a rise in a stimulation threshold of said electrode.

2. The electrode according to claim 1, wherein the polymer is a sulfonated polytetrafluoroethylene.

electrode is introduced into the tissues of the heart, the heart's muscular system is damaged, which in turn leads to the growth of connective tissue.

An example of a prior art implantable carbon electrode is set for the German Published Patent Application 28 42 318. It serves in particular as a stimulating electrode, for example as for a pacemaker. The surface of this electrode has a smooth coating of hydrophilic, ionic conducting plastic, whereby at least the surface of the plastic coating consists of material which is compatible with the body or blood. This plastic coating minimizes the energy losses occurring in the heart muscle as a result of a postoperative rise in the stimulation threshold. This is also believed to reduce clot formation at the electrode surface (which leads to increased initial and continuous stimulation thresholds).

The prior art also discloses a titanium electrode which is coated with platinum and which has a plug that lies behind the porous electrode surface. This plug is made of silicone rubber and contains a small amount (<1 mg) of the steroid dexamethasone sodium phosphate. (c.f.: Society for Biomaterials "Transactions 13th Annual Meeting", June 3-7, 1987, New York, pp 52, as well as PACE, vol. 11 (1988), pp. 214 to 219.) After the electrode has been exposed to body fluids (i.e. after implantation), the steroid is gradually released or eluted. This has been thought to increase the efficacy of the electrode. However, most of the steroid remains in the electrode for some time after implantation and is not quickly distributed into the surrounding tissue. Two years after implantation, 80% of the steriod remains in the electrode. One can extrapolate that 18% of the steroid would remain after 100 years.

In the prior art electrode, the availability of the steroid, i.e., its release from the electrode, is not only limited by the long time it takes, but--subject to the electrode design--it is limited locally (i.e. spatially) as well; i.e., it does not extend completely over the electrode surface. Moreover, it has been shown that as a result of adsorption at the surface of activated porous electrodes, organic silicon compounds or silicon adhesives can adversely effect the operation of the electrode, as they make the electrode surface water-repellent and thereby reduce the double-layer capacitance.

Therefore there is a need to provide an implantable stimulating electrode containing a steroid which both reduces the postoperative rise in the stimulation threshold and diminishes the growth of connective tissue, without any reduction in capacitance. Furthermore, the steroid should be uniformly delivered to the surrounding tissue within a short period of time to commence immediately after the implantation of the electrode.

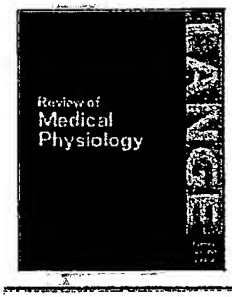
SUMMARY OF THE INVENTION

The present invention fills this need by utilizing an electrode whose surface has a thin coating of a hydrophilic polymer in which is embedded an anti-inflammatory steroid.

The porous electrode surface of the electrode of the present invention is provided with a thin layer of a polymeric plastic, into which is inserted a steroid that diffuses from this layer into the adjoining tissue. In this manner, the inflammatory process is suppressed and the process by which the electrode becomes incorporated in the tissues of the heart muscle is abetted. By means of the spatially uniform, dosed release of the steroid in the region around the electrode head, the surrounding tissue is supplied with steroid uniformly over a short distance. Consequently, the growth of connective tissue is reduced and thus the postoperative rise in the stimulation threshold is lessened. The steroid is then available when needed immediately after implantation. Within as few as two days following implantation, the steroid is nearly completely eluted. As a result of the measures according to the invention, the porous surface of the electrode is protected from impurities. However, the porous electrode surface suffers no loss of capacitance.

McGraw Hill Professional

Review of Medical Physiology



By William F. Ganong
Date March 8, 2005
Format Paperback, 928 pages
ISBN 0071440402 / 9780071440400

Overview

Ideal for self-assessment and USMLE Step 1 review. Provides a current and concise overview of mammalian and human physiology. Thoroughly revised and updated, examples from clinical medicine have been integrated throughout the chapters to illuminate important physiologic concepts. Features more than 700 illustrations and a self-study section with 630 multiple choice questions.

Table of contents

INTRODUCTION: THE GENERAL & CELLULAR BASIS OF MEDICAL PHYSIOLOGY

In unicellular organisms, all vital processes occur in a single cell. As the evolution of multicellular organisms has progressed, various cell groups have taken over particular functions. In humans and other vertebrate animals, the specialized cell groups include a gastrointestinal system to digest and absorb food; a respiratory system to take up O_2 and eliminate CO_2; a urinary system to remove wastes; a cardiovascular system to distribute food, O_2, and the products of metabolism; a reproductive system to perpetuate the species; and nervous and endocrine systems to coordinate and integrate the functions of the other systems. This book is concerned with the way these systems function and the way each contributes to the functions of the body as a whole.

This chapter presents general concepts and principles that are basic to the function of all the systems. It also includes a short review of fundamental aspects of cell physiology. Additional aspects of cellular and molecular biology are considered in the relevant chapters on the various organs.

GENERAL PRINCIPLES

Organization of the Body

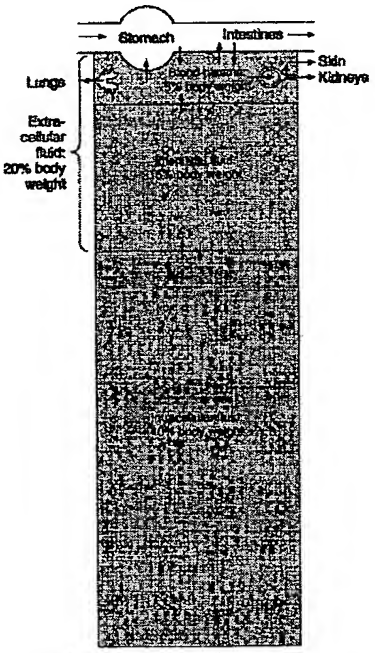
The cells that make up the bodies of all but the simplest multicellular animals, both aquatic and terrestrial, exist in an "internal sea" of **extracellular fluid (ECF)** enclosed within the integument of t animal. From this fluid, the cells take up O_2 and nutrients; into it, they discharge metabolic waste products. The ECF is more dilute than present-day seawater, but its composition closely resembles that of the primordial oceans in which, presumably, all life originated.

In animals with a closed vascular system, the ECF is divided into two components: the **interstitial fluid** and the circulating **blood plasma.** The plasma and the cellular elements of the blood, principally red blood cells, fill the vascular system, and together they constitute the **total blood volume.** The interstitial fluid is that part of the ECF that is outside the vascular system, bathing the cells. The special fluids lumped together as transcellular fluids are discussed below. About a third of the **total body water (TBW)** is extracellular; the remaining two thirds is intracellular **(intracellular fluid).**

Body Composition

In the average young adult male, 18% of the body weight is protein and related substances, 7% is mineral, and 15% is fat. The remaining 60% is water. The distribution of this water is shown in Figure 1–1.

Figure 1–1.



Body fluid compartments. Arrows represent fluid movement. Transcellular fluids, which constitute a very small percentage of total body fluids, are not shown.

The intracellular component of the body water accounts for about 40% of body weight and the extracellular component for about 20%. Approximately 25% of the extracellular component is in the vascular system (plasma = 5% of body weight) and 75% outside the blood vessels (interstitial fluid = 15% of body weight). The total blood volume is about 8% of body weight.

Measurement of Body Fluid Volumes

It is theoretically possible to measure the size of each of the body fluid compartments by injecting substances that will stay in only one compartment and then calculating the volume of fluid in whic the test substance is distributed (the **volume of distribution** of the injected material). The volume of distribution is equal to the amount injected (minus any that has been removed from the body metabolism or excretion during the time allowed for mixing) divided by the concentration of the substance in the sample. *Example:* 150 mg of sucrose is injected into a 70-kg man. The plasma sucrose level after mixing is 0.01 mg/mL, and 10 mg has been excreted or metabolized during the mixing period. The volume of distribution of the sucrose is

$$\frac{150\text{ mg} - 10\text{ mg}}{0.01\text{ mg/mL}} = 14{,}000\text{ mL}$$

Since 14,000 mL is the space in which the sucrose was distributed, it is also called the **sucrose space.**

Volumes of distribution can be calculated for any substance that can be injected into the body, provided the concentration in the body fluids and the amount removed by excretion and metabolism c be accurately measured.

Although the principle involved in such measurements is simple, a number of complicating factors must be considered. The material injected must be nontoxic, must mix evenly throughout the compartment being measured, and must have no effect of its own on the distribution of water or other substances in the body. In addition, either it must be unchanged by the body during the mixi period, or the amount changed must be known. The material also should be relatively easy to measure.

Plasma Volume, Total Blood Volume, & Red Cell Volume

B-4

Goodman & Gilman's The Pharmacological Basis of Therapeutics, 11th Edition

Laurence L. Brunton, editor-in-chief
John S. Lazo and Keith L. Parker, Associate Editors

Editors of the online edition:
Laurence L. Brunton, editor-in-chief
Associate Editors: Keith L. Parker, Iain L. O. Buxton, and Donald K. Blumenthal

Goodman & Gilman's Updates

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10/30/2006: Ranolazine: A New Antianginal Agent with a Novel Mechanism of Action Chaitman, Bernard R.

9/8/2006: Hot Topic: Warfarin Pharmacogenomics Rettie, Allan E.; Rieder, Mark J.

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of certain genes, including pro-opiomelanocortin (POMC) expression by corticotropes, is inhibited by glucocorticoid treatment. CBG, corticosteroid-binding globulin; GR, glucocorticoid receptor; S, steroid hormone; HSP90, the 90-kd heat-shock protein; HSP70, the 70-kd heat-shock protein; IP, the 56-kd immunophilin; GRE, glucocorticoid-response elements in the DNA that are bound by GR, thus providing specificity to induction of gene transcription by glucocorticoids. Within the gene are introns (*unshaded*) and exons (*shaded*); transcription and mRNA processing leads to splicing and removal of introns and assembly of exons into mRNA.

The receptors for corticosteroids are members of the nuclear receptor family of transcription factors that transduce the effects of a diverse array of small, hydrophobic ligands, including the steroid hormones, thyroid hormone, vitamin D, and retinoids. These receptors share two highly conserved domains: a region of approximately 70 amino acids forming two zinc-binding domains, called *zinc fingers,* that are essential for the interaction of the receptor with specific DNA sequences, and a region at the carboxyl terminus that interacts with ligand (the ligand-binding domain).

Although complete loss of glucocorticoid receptor (GR) function apparently is lethal, mutations leading to partial loss of GR function have been identified in rare patients with generalized glucocorticoid resistance (Bray and Cotton, 2003). These patients harbor mutations in the GR that impair glucocorticoid binding and decrease transcriptional activation. As a consequence of these mutations, cortisol levels that normally mediate feedback inhibition fail to suppress the HPA axis completely. In this setting of partial loss of GR function, the HPA axis resets to a higher level to provide compensatory increases in ACTH and cortisol secretion. Because the GR defect is partial, adequate compensation for the end-organ insensitivity can result from the elevated cortisol level, but the excess ACTH secretion also stimulates the production of mineralocorticoids and adrenal androgens. Because the mineralocorticoid receptor (MR) and the androgen receptor are intact, these subjects present with manifestations of mineralocorticoid excess (hypertension and hypokalemic alkalosis) and/or of increased androgen levels (acne, hirsutism, male pattern baldness, menstrual irregularities, anovulation, and infertility). In children, the excess adrenal androgens can cause precocious sexual development.

Glucocorticoid Receptor

The GR resides predominantly in the cytoplasm in an inactive form until it binds glucocorticoids (Figure 59–5). Steroid binding results in receptor activation and translocation to the nucleus. The inactive GR is complexed with other proteins, including heat-shock protein (HSP) 90, a member of the heat-shock family of stress-induced proteins; HSP70; and a 56,000-dalton immunophilin, one of the group of intracellular proteins that bind the immunosuppressive agents *cyclosporine* and *tacrolimus* (*see* Chapter 52 for a discussion of these agents). HSP90, through interactions with the steroid-binding domain, may facilitate folding of the GR into an appropriate conformation that permits ligand binding.

Regulation of Gene Expression by Glucocorticoids

After ligand binding, the GR dissociates from its associated proteins and translocates to the nucleus. There, it interacts with specific DNA sequences within the regulatory regions of affected genes. The short DNA sequences that are recognized by the activated GR are called *glucocorticoid responsive elements* (GREs) and provide specificity to the induction of gene transcription by glucocorticoids. The consensus GRE sequence is an imperfect palindrome (GGTACAnnnTGTTCT, where n is any nucleotide) to which the GR binds as a receptor dimer. The mechanisms by which GR activates transcription are complex and not completely understood, but they involve the interaction of the GR with transcriptional coactivators and with proteins that make up the basal transcription apparatus. Genes that are negatively regulated by glucocorticoids also have been identified. One well-characterized example is the pro-opiomelanocortin gene, whose negative regulation in corticotropes by glucocorticoids is an important part of the negative feedback regulation of the HPA axis. In this case, the GR appears to inhibit transcription by a direct interaction with a GRE in the *POMC* promoter. Other genes negatively regulated by glucocorticoids include genes for cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (NOS2), and inflammatory cytokines.

Although glucocorticoids and the GR are essential for survival, interactions of the GR with specific GREs apparently are not. These conclusions are supported by the findings that genetically engineered mice completely lacking GR function die immediately after birth, whereas mice harboring a mutated GR incapable of binding to DNA are viable. These observations imply that the critical function of GR involves protein–protein interactions with other transcription factors. Indeed, protein–protein interactions have been observed between the GR and the transcription factors NF-κB and AP-1, which regulate the expression of a number of components of the immune system (De Bosscher et al., 2003). Such interactions repress the expression of genes encoding a number of cytokines—regulatory molecules that play key roles in the immune and inflammatory networks—and enzymes, such as collagenase and stromelysin, that are proposed to play key roles in the joint destruction seen in inflammatory arthritis. Thus, these negative effects on gene expression appear to contribute significantly to the antiinflammatory and immunosuppressive effects of the glucocorticoids.

B-6

MOLECULAR CELL BIOLOGY

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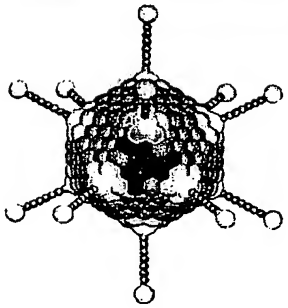

SCIENTIFIC
AMERICAN

Front cover photograph courtesy of J. Victor Small

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Library of Congress Cataloging-in-Publication Data

Darnell, James E.
Molecular cell biology.

Includes bibliographies and index.
1. Cytology. 2. Molecular biology. I. Lodish, Harvey F. II. Baltimore, David. III. Title.
[DNLM: 1. Cells. 2. Molecular Biology. QH 581.2 D223m]
QH581.2.D37 1986 574.87'6042 86-1881
ISBN 0-7167-1448-5
ISBN 0-7167-6001-0 (international student ed.)

Printed in the United States of America

Scientific American Books is a subsidiary of Scientific American, Inc.
Distributed by W. H. Freeman and Company, 41 Madison Avenue, New York, New York 10010

1 2 3 4 5 6 7 7 8 9 0 KP 4 3 2 1 0 8 9 8 7 6

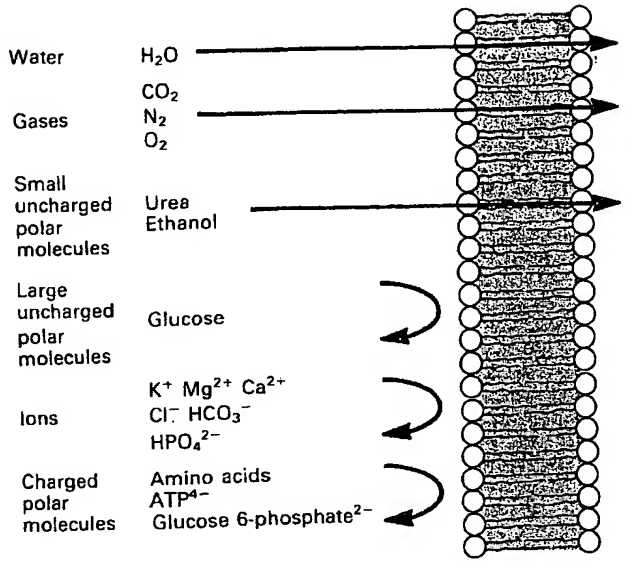

Figure 15-1 A pure artificial phospholipid bilayer is permeable to water, to small hydrophobic molecules, and to small uncharged polar molecules. It is not permeable to ions or to large uncharged polar molecules. Figure 14-7 demonstrates how such artificial membranes are prepared. Using an apparatus in which a small phospholipid bilayer separates two aqueous compartments (Figure 14-7), one can easily measure the permeability of the membrane to various substances: simply add a small amount of the material to one chamber and measure its rate of appearance in the other. The use of radioactive substances such as [^{14}C]glucose or radiolabeled Na^+ greatly facilitates such experiments.

transport molecules within the plasma membrane. Two different types of mechanisms have evolved to allow such molecules to enter or leave the cell.

First, ions and small molecules, including sugars and amino acids, are transported across the lipid of the plasma membrane. Each of various specific integral membrane proteins, termed *permeases*, facilitates the transport of only a limited range of molecules. Because different cell types require different mixtures of low-molecular-weight compounds, the plasma membrane of each cell type contains its own individually tailored battery of permease proteins. Similarly, the membrane surrounding each type of subcellular organelle contains a specific set of permeases that allow only certain molecules to cross it. Indeed, it has become evident in recent years that virtually all the permeability of membranes to small molecules is both facilitated and regulated in various ways by proteins within the membrane. These various mechanisms are discussed extensively in the first part of this chapter.

Second, protein molecules and larger particles enter the cell by endocytosis and phagocytosis (see Figure 5-38). Small regions of the plasma membrane surround the macromolecule or particle required by the cell, then the membrane and its contents are internalized by the cell, forming an intracellular vesicle. A broad array of nutrients, viruses, and particles enter the cell this way. The processes are discussed in detail in the last section of this chapter.

Transport and the Intracellular Ionic Environment

An important function of the plasma membrane is to maintain an ionic composition in the cytosol very different from that of the surrounding fluid. In both vertebrates and invertebrates, for example, the concentration of sodium ion is about 10 to 20 to 40 times higher in the blood than within the cell. The concentration of potassium ion is the reverse, generally 20 to 40 times higher inside the cell (Table 15-1). The generation and maintenance of such gradients on either side of a semipermeable membrane requires the expenditure of a great deal of energy.

Transport across a membrane may be passive or active. *Passive transport* is a type of diffusion in which an ion or molecule crossing a membrane moves down its electrochemical or concentration gradient. No metabolic energy is expended in passive transport. In *simple diffusion*, pas-

Table 15-1 Typical ionic concentrations in invertebrates and vertebrates

	Cell	Blood (m*M*)
SQUID AXON*		
K^+	400 m*M*	20
Na^+	50 m*M*	440
Cl^-	40–150 m*M*	560
Ca^{2+}	0.3 μ*M*	10
X^{-}†	300–400 m*M*	
MAMMALIAN CELL		
K^+	139 m*M*	4
Na^+	12 m*M*	145
Cl^-	4 m*M*	116
HCO_3^-	12 m*M*	29
X^{-}†	138 m*M*	9
Mg^{2+}	0.8 m*M*	1.5
Ca^{2+}	<1 μ*M*	1.8

*The large nerve axon of the squid is chosen as an example of an invertebrate cell, as it has been used widely in studies of the mechanism of conduction of electrical impulses.

†X^- represents proteins, which have a net negative charge at the neutral pH of blood and cells.

Control by Pulse Parameters of Electric Field-Mediated Gene Transfer in Mammalian Cells

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ABSTRACT Electric field-mediated gene transfer in mammalian cells (electrotransformation) depends on the pulsing conditions (field intensity, pulse duration, number of pulses). The effect of these parameters was systematically investigated using the transient expression of the chloramphenycol acetyltransferase and the β-galactosidase activities in Chinese hamster ovary cells. Pulsing conditions inducing reversible permeabilization of the cell plasma membrane are not sufficient to induce gene transfer. The plasmid must be present during the electric pulse if it is to be transferred across the membrane into the cytoplasm. Only the localized part of the cell membrane brought to the permeabilized state by the external field is competent. Pulse duration plays a key role in the magnitude of the transfer. The field induces a complex reaction between the membrane and the plasmid that is accumulated at the cell interface by electrophoretic forces. This leads to an insertion of the plasmid, which can then cross the membrane.

GLOSSARY

E	electric field
I	impermeant state of the membrane
P	permeabilized state of the membrane
Tr	transformation yield
T	pulse duration
N	pulse number
u	electrophoretic mobility
L	electrophoretic displacement

INTRODUCTION

Transfer of foreign information in the genome of cells is a key problem in cell biology and biotechnology. This is crucial in the study of eucaryotic cells where there is no spontaneous transfer such as exists in bacteria. Transfer is obtained by chemical or viral approaches, although with many limitations (Malissen, 1990). A physical approach using the effect of electric field pulses on cells was pioneered in the early 1980s (Neumann et al., 1982). Electrotransformation is now routinely used in molecular biology (Potter, 1992) because of the broader range of cells that are sensitive to the electric technique as compared to other techniques. Large volumes of cells can be routinely treated (Rols et al., 1992). But very few studies have focused on the electric field-mediated mechanism of gene transfer, except in the case of bacterias (Xie and Tsong, 1990, 1992; Xie et al., 1990, 1992; Eynard et al., 1992). It is proposed that in the case of mammalian cells the plasmid crosses the membrane during the pulse due to the induction of an electropermeabilized state and under the effects of electrophoretic forces associated with the external field (Klenchin et al., 1991; Sukharev et al., 1992). But this conflicts with the results on *Escherichia coli* where no role for electrophoretic forces was observed. Indeed very few experimental results have been gathered on the reversible organization of the permeabilized membrane. Different theoretical descriptions have nevertheless been proposed: breakdown (Crowley, 1973), pores (see for reviews Neumann et al., 1989; Chang et al., 1992) or mismatches (Cruzeiro-Hanssen and Mouritsen, 1989). ^{31}P NMR studies have shown that the structural organization of the membrane phospholipids is affected inducing a fusogenic character in the electropermeabilized cell membrane (Sowers, 1986; Teissié and Rols, 1986; Lopez et al., 1988). Reorganization of the membrane/solution interface was proposed to be a key step in the induction of permeabilization (Rols and Teissié, 1990a). Electron microscopy investigation under isoosmolar conditions only revealed very short lived electrocracks (Stenger and Hui, 1986) and longer lived eruptions of villi (Escande et al., 1988; Gass and Chernomordik, 1990). "Volcano"-shaped pores were detected in red blood cells under hypoosmolar conditions (Chang and Reeves, 1990), but were correlated with the hemoglobin induced osmotic swelling (Chernomordik, 1992). The time dependence of the eruption of these pores was very different from that of the conductance changes (Kinosita and Tsong, 1979; Hibino et al., 1993). A kinetic model of the induction of electropermeabilization was proposed where a multistep process comprising induction steps (nucleation) followed by fast collective coalescence of the defects in ramified cracks was present (Sugar et al., 1987). Qualitative investigations of the process demonstrated that the magnitude of the field controlled the geometrical definition of the permeabilized part of the cell surface (Schwister and Deuticke, 1985) but that the cumulated pulse duration determined the local magnitude of the alteration (Rols and Teissié, 1990b).

In the present study, the effect of the different parameters controlling both transient electrotransformation and electropermeabilization of Chinese hamster ovary cells

Received for publication 2 August 1993 and in final form 15 October 1993.
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0006-3495/94/02/524/08 $2.00

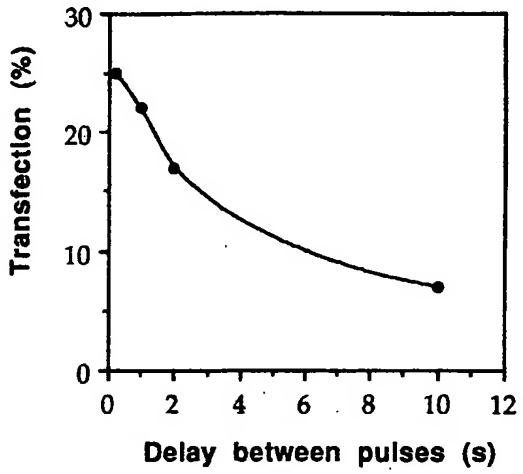

FIGURE 7 Electrotransformation as a function of the period between pulses. The DNA-cell mixture was pulsed ten times at different frequencies with fields of 0.8 kV/cm lasting 5 ms. The level of β-galactosidase activity (the percentage of blue-stained cells) is plotted as a function of the delay between the pulses.

200 μl. In the electrotransfection experiments, 5 pulses lasting 1 ms were applied at a frequency of 1 Hz and at 0.9 kV/cm intensity. A 60-fold increase in gene transfer was obtained with the electric approach with a 40% associated loss of viability.

Control of DNA transfer by the plasmid

The level of transfered activity was related to the concentration of added plasmid as shown in Fig. 8. The amount of added plasmid was changed by diluting the stock DNA with Tris-EDTA buffer. The viability of pulsed cells was affected by the concentration of DNA (Fig. 8). For concentrations ranging from 1 to 13 μg/ml, the viability remained unchanged but decreased for higher concentrations. This suggests a synergic effect of DNA and electric pulses on cell viability. This was confirmed by electropulsing cells in a 1 mg/ml Salmon sperm DNA. All pulsed cells were lysed 24 h after pulsing.

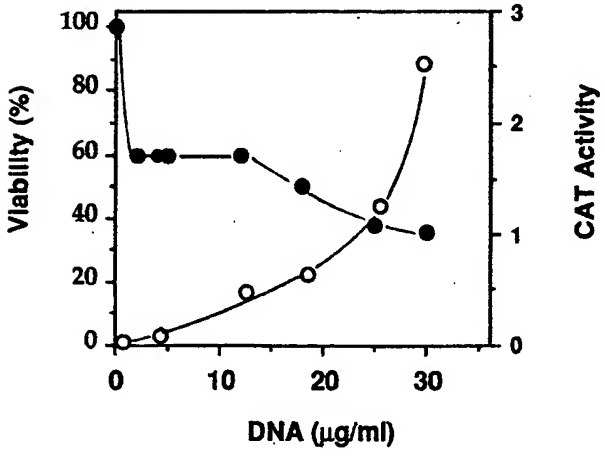

FIGURE 8 Electrotransformation and viability of pulsed CHO cells as a function of the amount of PSV2CAT plasmids. The DNA-cell mixture was pulsed five times with fields of 1.125 kV/cm lasting 1 ms. The level of CAT activity (counts/mg protein) is plotted as a function of the concentration of coding plasmids, no carrier DNA being present.

Different pre- and post-pulse incubation times were checked. Results are in Table 1. The conclusions are that both pre and post incubations improve the level of transformation. But the main fact is that DNA must be present during the pulse. By adding the plasmid only 2 s after pulsing, the shortest period we can operate, we observed that the cell suspension did not undergo any transformantion, in agreement with previous observations (Winterbourne et al., 1988; Klentchin et al., 1991).

DISCUSSION

The purpose of this work was to investigate the mechanism of electrotransformation. Up to now, most experiments dealing with this approach to gene transfer have assumed that the plasmid crossed the membrane due to electrically induced permeability under the effect of electrophoretic forces mediated by the external field (Klenchin et al., 1991; Sukharev et al., 1992). It was implicitly assumed that electropermeabilization and electrotransformation occured through similar processes. The mechanisms of such processes are still to be elucidated although recent models based on experimental data have been proposed (Dimitrov and Sowers, 1990).

Our results indeed indicate that electric field pulses inducing permeabilization are needed to mediate the gene transfer. The intensity of the field must be larger than the characteristic threshold required to permeabilize the cell membrane locally. If one describes electropermeabilization as a local reaction bringing the membrane from the native impermeant state I to a permeabilized one P as observed by videomicrosopy (Kinosita et al., 1988; Hibino et al., 1993)

$$I \xrightarrow[E]{} P \qquad (3)$$

in which E is the electric pulse, then electrotransformation can only occur in the part of the cell surface which can be

TABLE 1 Electrotransformation of CHO cells: effect of the timing of the DNA-cell mixing

Conditions	Counts/ mg protein
No pulse	13
Pulse (standard conditions)	1715 (±5)
DNA added 2 S after pulsing	17 (±2)
DNA added 30 S after pulsing	33 (±7)
DNA added 1 min after pulsing	50
DNA added 2 min after pulsing	23
No carrier DNA	2000
Cells were pulsed just after adding DNA	900
The DNA-cell mixture was diluted just after pulsing	740

0.9×10^6 cells were mixed with 5.5 μg of PSV2CAT and 10 μg of salmon sperm DNA in a final volume of 200 μl of PBA. 5 pulses lasting 1 ms were applied at a frequency of 1 Hz with a 0.9 kV/cm intensity.

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Respectfully submitted,

21 Feb 07
Date

Alan King, D.V.M., Ph.D.